

Life-history trait variation in tadpoles of the warty toad in response to pond drying

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Abstract

We explored the response to habitat desiccation in tadpoles of the warty toad *Rhinella spinulosa* in a manipulative field experiment. We built an artificial pond system with two desiccation levels (high and low) and populated with tadpoles at Gosner stage 25. Each treatment was replicated six times. We measured the survival, size and age at metamorphosis, development rate and hind limb length in metamorphs. The results showed that tadpoles from the high desiccation ponds accelerated their development, reaching metamorphosis at an earlier age than tadpoles from the low desiccation ponds. Survival, size at metamorphosis and hind limb length were not different between treatments. This experiment demonstrated that tadpoles of *R. spinulosa* accelerate their development in response to habitat desiccation. Such plasticity may allow them to avoid mortality in short duration ponds. No evidence for a trade-off between development time and size at metamorphosis was found in this experiment. We suggest that factors such as initial tadpole density and nutritional quality of food would contribute towards determining whether metamorphosis occurs at the developmental threshold or at a larger size.

Introduction

Phenotypic plasticity refers to the phenotypic variation induced by all forms of environmental change (Stearns, 1989). Organisms that undergo a complex life cycle are interesting models to evaluate the role of phenotypic plasticity in the ecology and evolution of variable environments [i.e. insects (Gotthard, 1998), crustaceans (Hentschel & Emler, 2000), fish (Reznick, 1990) and amphibians (e.g. Rowe & Ludwig, 1991; Morey & Reznick, 2004; Rudolf & Rodel, 2007)]. Temporary ponds are classic examples of heterogeneous environments (Newman, 1992). In these systems, making the transition from an aquatic to a terrestrial habitat at the appropriate size and time is crucial to fitness (Rowe & Ludwig, 1991; Johansson, Hjelm & Giles, 2005).

Life-history responses of amphibian larvae have received considerable attention in the literature (Leips & Travis, 1994). An important body of theoretical and experimental work has been developed to understand the larval response variation to biotic and abiotic factors (Wilbur & Collins, 1973; Wilbur, 1987; Leips, McManus & Travis, 2000). Wilbur & Collins (1973) developed a model, that proposes that timing to metamorphosis would be related to recent growth history, predicting that when the larval growth rate

increases, the development rate is retarded to take advantage of the improved growth conditions. In contrast, development rate increases if growth rate decreases, in order to escape to suboptimal environments. Travis (1984) postulated that growth and development rates are dissociated only at the beginning of the larval period, according to which the development rate would be set early in the larval period and posterior changes in the growth rate could operate only in the size at metamorphosis.

More recent models have incorporated predictions related to the influence of time constraints (e.g. habitat duration and seasonality) on development time and, consequently, on the age and size at metamorphosis (Gomez-Mestre & Buchholz, 2006; Rudolf & Rodel, 2007). When the risk of mortality in the larval environment increases as a function of time, a younger age at metamorphosis may be favored in spite of costs associated with a smaller body size (Werner, 1986; Brady & Griffiths, 2000). For example, many amphibian species that undergo larval development in temporary ponds accelerate their development rate to avoid mortality due to desiccation (Wilbur, 1987; Newman, 1988, 1992; Laurila & Kujasalo, 1999; Loman, 2002). On the other hand, there are some species that breed in temporary ponds but do not respond to drying conditions, decreasing the larval period [i.e. *Scaphiopus multiplicatus* (Pfennig, Mabry

& Orange, 1991); *Scaphiopus holbrooki* (Wilbur, 1987) and *Bufo punctatus* (Tevis, 1966)], suggesting different phenotypic plastic responses. Thus, the amphibian model is exceptionally useful for examining the theoretical predictions related to understand how the duration of temporary ponds has shaped the evolution of phenotypic plasticity.

Field studies conducted to explore amphibian life-history traits in response to pond drying are not as common as laboratory studies (e.g. Newman, 1989; Tejedo & Reques, 1994; Leips *et al.*, 2000; Loman, 2002; Morey & Reznick, 2004). The advantage of field studies is that they are more realistic than laboratory experiments (Diamond, 1986). Regarding the warty toad *Rhinella spinulosa*, Márquez-García *et al.* (2009), in a field experiment, detected variation in size and age at metamorphosis in toadlets belonging to ponds with different desiccation levels. However, the methodology used in that study did not allow them to establish clearly which factors were responsible for the positive relation between hydroperiod and size at metamorphosis, as there was no control over several variables that may have affected the observed responses, such as initial density. In this study, we examined the plasticity response of *R. spinulosa* to pond drying by means of a manipulative field experiment. This approach allows the regulation of independent variables as well as manages the replication and the number of experimental manipulations (Diamond, 1986).

The warty toad *R. spinulosa* in central Chile breeds in temporary ponds that may experience high variability in pond duration, and where catastrophic mortalities are common events. We designed a manipulative field experiment to evaluate whether pond drying promotes phenotypic plasticity in the life-history traits of this species by constructing an artificial pond system with two desiccation levels (high and low). We measured survival, size and age at metamorphosis, development rate and hind limb length in metamorphs grown in these experimental ponds.

Materials and methods

Study species

Rhinella spinulosa Wiegmann, 1835 (Anura: Bufonidae) has a wide geographic distribution in Chile; it extends from the Altiplano of Chile, Argentina, Bolivia and Peru to the Andean central Chile (33°S) (Velooso *et al.*, 1982; Velooso & Navarro, 1988; Méndez *et al.*, 2004). A common garden experiment with tadpoles from Farellones (33°20'48.3"S; 70°18' 51.2"W) found that these larvae had shorter times to metamorphosis and greater growth rates compared with northern populations, which do not experience pond desiccation (Méndez & Correa-Solis, 2009). These antecedents suggest local adaptation to the natural desiccation regime of the ponds in central Chile and led us to study the existence of phenotypic plasticity as a function of desiccation in this area.

Artificial ponds and desiccation simulation

During November 2007, we built a system of 12 artificial ponds in an enclosed field located at La Parva, in the Andes mountain range of central Chile (33°19'55.8"S; 70°17'56.9"W, altitude: 2601 m a.s.l.). All ponds were 1 m in diameter and to simulate the profile of a natural pond, they were constructed with a slight slope on the sides. This experimental design allows tadpoles to pick their preferred depth in each pond and avoid hypoxic water. Ponds were lined with plastic to avoid water drainage and were filled with water from a nearby well. Figure 1 shows the spatial arrangement of the artificial ponds.

Ponds were not covered but we checked the system every day and we did not observe any predation on tadpoles. In fact, we did not observe either invertebrates or terrestrial predators in the study area during the entire experimental period. As we have been following this area for 8 years, and we have never observed vertebrates eating larvae, we exclude the possible effect of this kind of predator in our experimental ponds.

Pond desiccation treatment consisted of two levels: high and low, and they were differentiated by their initial maximum depth. High desiccation ponds were set as 15 cm maximum depth, whereas low desiccation ponds were set as 30 cm. Each treatment was replicated six times. The water in the high desiccation treatment was maintained at its maximum depth for 2 weeks after the start of the experiment on December 12 and then was allowed to evaporate. These short-duration ponds dried 65 days after the start of the experiment. The low desiccation treatment was maintained with water at its maximum depth for 65 days and was then allowed to evaporate, drying up 100 days after the start of the experiment (on March 21). These pond durations fell

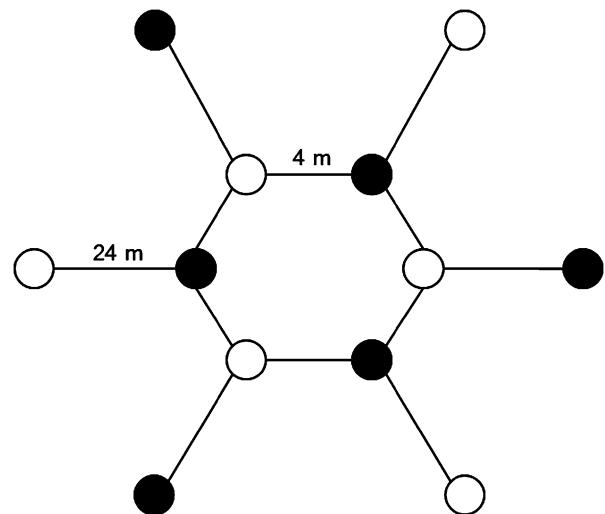


Figure 1 Experimental layout of artificial ponds with different desiccation levels. All ponds were 1 m in diameter. Low desiccation ponds (●) were set as 30 cm maximum depth; high desiccation ponds (○) were set as 15 cm maximum depth.

Table 1 Results of partial Mantel tests performed for (A) the life-history traits distance matrix (dependent variables), (B) the Euclidean distances matrix and (C) the experimental design matrix

Dependent variable	$r(AB,C)$	P
Survival to metamorphosis	0.053	0.694
Survival of metamorphs and tadpoles	0.066	0.583
Size at metamorphosis	0.190	0.127
Age at metamorphosis	-0.077	0.514

All analyses were performed with the program XLSTAT version 2009.1.02 (Addinsoft, 2005), using 10 000 permutations.

within the range observed in natural ponds located in the study area (Table 1, Márquez-García *et al.*, 2009).

Experimental procedure

We collected three clutches of *R. spinulosa* in a creek from the study area on the same day in November. These eggs were transported to the laboratory, where they were maintained aerated at 20 °C. When tadpoles reached Gosner's stage 25 (Gosner, 1960), we randomly assigned them to 12 groups of 167 larvae each. For this purpose, each clutch was divided into 12 equal parts that were mixed, forming groups with tadpoles from the three clutches. This procedure allowed us to reduce the possible egg-size-mediated maternal effects and also for blending out genetic differences among clutches. Each group of tadpoles was randomly assigned to each artificial pond during the first day of the experiment. Adequate oxygenation of the water was maintained by bubbling the ponds with an air pump for one hour every two days. Tadpoles were fed *ad libitum* with the microalgae *Spirogyra* sp., which is a main food item in natural conditions. We measured the temperature of ponds every hour from 09:00 to 19:00 h using a WTW digital thermometer (WTW Inc., Gold River, CA, USA), during the whole study period.

Development time

To determine the developmental stage of tadpoles from each treatment, we took weekly samples. We collected 10 tadpoles per pond, whose developmental stage was determined using Gosner's table (Gosner, 1960) and they were then returned to the pond. Thus, we obtained the frequencies of developmental stages over time for each treatment.

Size and age at metamorphosis

After the first metamorph (Gosner's stages 44 or 45) was discovered (about January 10), ponds were checked daily and all metamorphs found in each pond were collected. They were anesthetized with 0.02% MS-222 (tricaine methanesulfonate) following Anholt, Negovetic & Som (1998) and then we measured the snout-vent length (SVL) and the hind limb length with a digital caliper (0.02 mm precision). Because it has been reported that the hind limb length varies

independent of body size as a result of pond desiccation (e.g. Newman, 1989; Richter-Boix, Llorente & Montori, 2006; Márquez-García *et al.*, 2009), we decided to measure this trait. Once individuals recovered from the anesthesia, we returned them to their original ponds.

To avoid the recapture of measured individuals, while anesthetized, they were marked with a fluorescent polymer developed by Northwest Marine Technology Inc. (Shaw Island, WA, USA) called a visible implant elastomer, which is biologically inert. Observations with marked toadlets in the same pond system showed that they remained in their pond after metamorphosis for 2 days (Kraemer, 2009). Thus, we are confident that we measured the majority of metamorphs and that they belonged to the ponds where they were found.

The age at metamorphosis (number of days to reach Gosner stage 44 or 45 since the start of the experiment) was registered for each collected metamorph. Also, as ponds dried, the surviving tadpoles were counted. Survival was therefore expressed as (1) survival to metamorphosis and (2) survival of both metamorphs and tadpoles at the end of each treatment.

Statistical analyses

To assess the occurrence of spatial autocorrelation among the experimental units (ponds), we performed partial Mantel tests using: (a) the distance matrix of the measurements of dependent variables among replicates (survival, size and age at metamorphosis), (b) the matrix of Euclidean distances between ponds and (c) the experimental design matrix (which expresses the differences in the treatments to which the experimental units were exposed). Thus, we tested whether survival, size and age at metamorphosis were spatially correlated while the treatment effects were held constant. These analyses were performed with XLSTAT program version 2009.1.02 (Addinsoft, 2005), using 10 000 permutations. The results showed that the spatial locations of the ponds did not affect the measured variables (Table 1), suggesting that there is no spatial dependence among replicates.

To compare the frequency distribution of development stages between treatments in the several dates sampled, we used the Kolmogorov-Smirnov test. The percentage of metamorphs per week, the age at metamorphosis and survival were compared between desiccation treatments using the Mann-Whitney *U*-test. Student's *t*-test was used to examine the treatment effect on size at metamorphosis. Hind limb length was analyzed with a univariate ANCOVA using SVL as a covariate and desiccation level as an independent variable. For parametric tests, the data were transformed to \log_{10} in order to satisfy the assumptions of normality and homoskedasticity. The variation of mean, maximum and minimum temperatures between treatments was analyzed separately by Mann-Whitney *U*-tests, as the data could not be transformed to a normal distribution. All statistical analyses were performed using the software STATISTICA 6.0 (Statsoft, 2001).

Results

Water temperature

Table 2 shows the average of daily, minimum and maximum temperatures for desiccation level. The high desiccation level had a greater mean temperature than the low desiccation level ($U = 73\,691$, $P < 0.001$; Mann–Whitney U -test). High desiccation ponds had lower daily minimum temperatures as well as higher maximum temperatures. The difference was not significant for the minimum ($U = 379$, $P = 0.11$; Mann–Whitney U -test), but it was for the maximum ($U = 231$, $P < 0.001$; Mann–Whitney U -test).

To evaluate whether size and age at metamorphosis were correlated with the mean temperature and/or the thermal oscillation of the ponds, we performed partial Mantel tests keeping the treatment effect constant. The correlations were not significant either for size ($r = -0.081$, $P = 0.527$) or for the age at metamorphosis ($r = 0.015$, $P = 0.908$) respecting to the mean temperature of each pond. There was a significant correlation between thermal oscillation and age at metamorphosis ($r = 0.282$, $P = 0.025$), while size was not significant ($r = -0.103$, $P = 0.403$).

Survival

The survival to metamorphosis was low in the system; it ranged between 5 and 33%. When the survival of both metamorphs and tadpoles was considered, survival ranged between 18 and 56%. There were no differences between

treatments for both kinds of survival ($U = 15$, $P = 0.63$ and $U = 10$, $P = 0.2$; Mann–Whitney U -test, respectively).

Development and age at metamorphosis

The frequency distributions of developmental stages were different between treatments starting from the second date sampled (Fig. 2). On January 4, there were no differences between desiccation levels ($P > 0.1$; Kolmogorov–Smirnov test), but in the following samples, development was significantly more advanced in tadpoles from the high desiccation treatment (January 14, $P < 0.05$; January 25, $P < 0.001$; February 1, $P < 0.005$; Kolmogorov–Smirnov tests). The mean age at metamorphosis in the high desiccation level was 53 ± 0.8 days ($n = 136$), while in the low desiccation level, it was 67 ± 1.4 days ($n = 157$); this difference was statistically significant ($U = 5531$, $P < 0.001$; Mann–Whitney U -test) (Fig. 3). Finally, the percentage of metamorphs per week was significantly greater for high desiccation ponds (Fig. 4; $U = 2.00$, $P = 0.028$; Mann–Whitney U -test), especially during the last two weeks of the high desiccation treatment.

Size at metamorphosis

Size at metamorphosis (SVL) was not different between treatments ($t_{290} = 1.04$, $P = 0.8$). The mean SVL for the high desiccation treatment [$10.83 \text{ mm} \pm 0.06$ ($n = 136$)] was similar to the low desiccation treatment [$10.78 \text{ mm} \pm 0.05$ ($n = 157$)]. No effect of desiccation level was observed in hind limb length (ANCOVA, $F_{1,290} = 1.16$, $P = 0.28$).

Discussion

Many authors have suggested that when the risk of mortality in the larval environment increases over time, as in temporary ponds, a younger age at metamorphosis may be favored in spite of the costs associated with a smaller body size (Werner, 1986; Rowe & Ludwig, 1991; Brady & Griffiths, 2000; Rudolf & Rodel, 2007). Our results show that larvae of *R. spinulosa* are able to adjust their

Table 2 Mean, minimum and maximum temperatures for the two desiccation levels

Treatment	Water temperature (°C)		
	Mean	Minimum (09:00 h)	Maximum (14:00 h)
High desiccation	22.57 ± 0.32	9.38 ± 0.28	28.32 ± 0.55
Low desiccation	19.37 ± 0.25	9.93 ± 0.33	23.00 ± 0.42

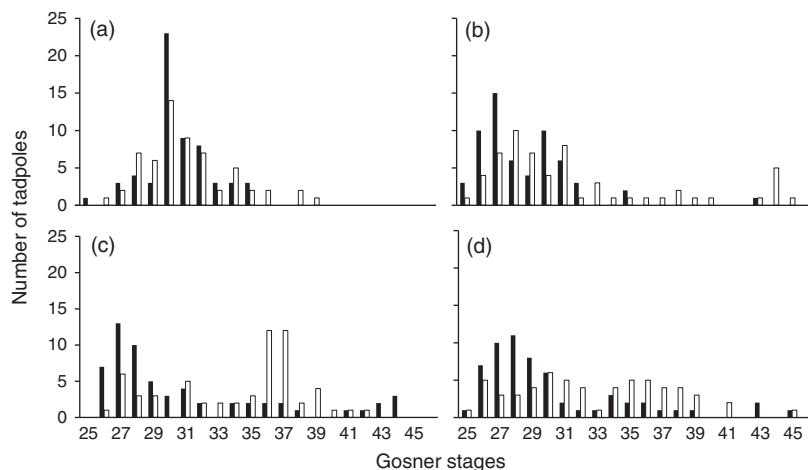


Figure 2 Frequency distributions of developmental stages in tadpoles from high desiccation (□) and low desiccation treatments (■). Samples taken on: (a) January 4, (b) January 14, (c) January 25 and (d) February 1. Differences between treatments were observed from the second date sampled [(b) $P < 0.05$, (c) $P < 0.001$, (d) $P < 0.005$; Kolmogorov–Smirnov tests].

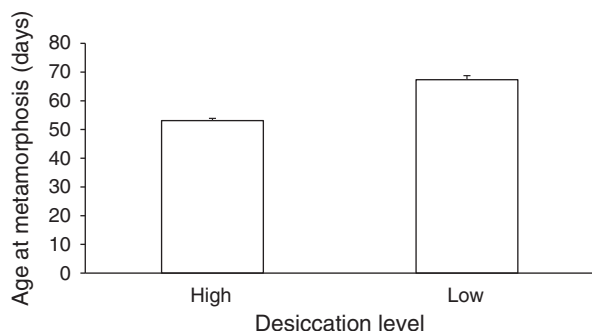


Figure 3 Mean age (\pm SE) at metamorphosis for the two desiccation levels. Tadpoles from the high desiccation treatment reached metamorphosis at an earlier age than tadpoles from the low desiccation treatment ($P < 0.001$; Mann–Whitney U -test).

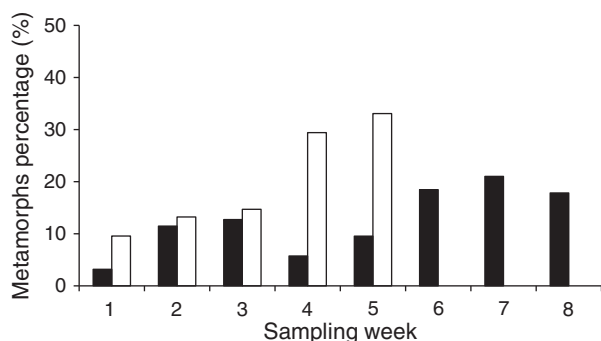


Figure 4 Percentage of metamorphs over time, in the high desiccation (\square) and low desiccation treatments (\blacksquare). During the weeks in which both treatments co-existed, a greater number of metamorphs emerged from the high desiccation ponds ($P < 0.05$; Mann–Whitney U -test).

development rate in response to drying conditions, a plasticity that would allow them to avoid mortality in short-duration ponds.

Both field and laboratory experiments conducted in many amphibian species have reported a similar trend, showing that prolonged larval periods are associated with greater size at metamorphosis (e.g. Newman, 1989; Morey & Reznick, 2004; Márquez-García *et al.*, 2009). In our experiment, the tadpoles of *R. spinulosa* accelerated their development in response to habitat desiccation, reaching metamorphosis at an earlier age but at a size similar to those tadpoles that delayed their metamorphosis. These results do not agree with the predictions of Travis (1984), because we observed an early acceleration in the development rate of this species in high desiccation ponds (Fig. 2), and this acceleration did not affect the size at metamorphosis. This response has been observed in natural and experimental ponds associated with low larval densities (e.g. Tejedo & Reques, 1994; Loman, 2002). Tejedo & Reques (1994) found that in the anuran *Bufo calamita*, the hydroperiod effect varied as a function of

larval density. In their study, only at high densities did long-duration ponds have heavier metamorphs than short-duration ponds. The initial larval densities in our experiment (3.34 and 1.67 larvae L^{-1}) were low compared with the natural densities found in this species. We estimate that natural densities should range from 10 to 30 larvae L^{-1} (not published data), which is an order of magnitude greater than in our experimental ponds. Therefore, it is probable that the size at metamorphosis observed has arisen due to the low initial larval density assigned to the ponds. In fact, data from the 2008–2009 season showed that increasing the initial larval density to 12.7 larvae L^{-1} in the same artificial ponds produced the expected pattern, that is individuals that delayed metamorphosis in long-duration ponds have a greater size at metamorphosis (Kraemer, 2009).

The prediction of the threshold model proposed by Wilbur & Collins (1973) and later formalized by Day & Rowe (2002) may be another plausible explanation for the size at metamorphosis observed in our experiment. Probably, the nutritional requirements of the tadpoles contributed to trigger metamorphosis at the developmental threshold in both treatments, which is the minimum size or condition that a developing organism must have reached before a life-history transition can occur. In our experiment, tadpoles only had the alga *Spirogyra* sp. as a main food, while under natural conditions, tadpoles also have the layer of mud at the bottom of the pond, from which they may obtain nutrients necessary to growth. It has been documented for several amphibians that food quality modulates the response of life-history attributes, including size at metamorphosis (review of Kupferberg, 1997; Wassersug, 1997). Benavides *et al.* (2005) showed that in tadpoles of *R. spinulosa*, the diet nutritional quality has a positive influence on assimilation efficiency and on metamorphic size. Thus, it is probable that the ‘mono-diet’ of *Spirogyra* sp. has affected the size at metamorphosis observed in both treatments, leading us to suppose that independent factors to habitat desiccation contribute to determine whether metamorphosis occurs at the developmental threshold or at a larger size.

During the weeks in which both treatments co-existed, a greater number of metamorphs emerged from the high desiccation ponds (Fig. 4). These larvae accelerated their development starting from the second week of desiccation (Fig. 2), at which time they probably detected specific cues associated with desiccation (see Newman, 1992). Although these specific cues remain unknown, one candidate is water temperature (Newman, 1989; Morey & Reznick, 2004). Our results showed that ponds with high desiccation had higher mean and maximum temperatures, and consequently had greater daily temperature fluctuations. The results of the partial Mantel tests suggest that the effect of treatments on the age at metamorphosis is correlated with the thermal oscillation of each pond, but not with the mean temperature. Thus, daily variance in water temperature may be a reliable signal to the imminent drying of the habitat of *R. spinulosa*. Accordingly, common garden studies will be required to determine whether temperature or other changes related to desiccation are necessary to induce the adaptive plasticity or

whether this species is able to respond to the temporal reduction in water volume *per se*.

Hind limb length is a functionally important trait in anurans (Emerson, 1978) and its variation can be explained by proximal (Blouin & Brown, 2000) and historical causes (Gomez-Mestre & Buchholz, 2006). Under natural conditions, organisms that delay metamorphosis have longer size-adjusted tibiofibulas and are able to jump further than individuals that accelerate development (Ficetola & De Bernardi, 2006). Therefore, the environment may induce a morphometric variation by affecting growth and differentiation rates (Blouin & Brown, 2000). Although the desiccation level in our artificial ponds affected the differentiation rates of the tadpoles, it did not induce a morphometric variation in the hind limb length. In contrast, the results of Márquez-García *et al.* (2009) showed that desiccation affected both hind limb length and size at metamorphosis in *R. spinulosa*. Differences in the growth rates are presumably responsible for differences in toadlet morphology observed in both studies.

Temporal habitat constraints promote a variation in life-history traits in tadpoles of the warty toad (*R. spinulosa*). The experimental approach used in this study allowed the identification of variables that help us to explain the observed variation. Undoubtedly, the addition of new variables in the experimental design, such as food quality and larval density, would allow a better understanding of the trends observed. Also, measuring other variables that are correlated with the decrease in water volume in natural conditions, such as water chemistry, may allow the determination of the cues that tadpoles detect to habitat imminent desiccation.

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